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TITLE: Enhancing the Phagocytic Clearance of Apoptotic Cells to Control Breast Carcinoma Progression

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14. ABSTRACT Macrophages have emerged as a key cell type influencing the initiation, progression and metastasis of breast cancer. Their impact on carcinogenesis is largely understood through their role in promoting a pro- or anti-inflammatory milieu. The phagocytosis of apoptotic cells by macrophages, a chief function of these cells, greatly influences the inflammatory status of macrophages. Despite the abundance of both macrophages and apoptotic cells in mammary tumors, little is known about how these cells interact in the tumor environment. Understanding how macrophages respond to apoptotic cells during the engulfment process should reveal important information on how this critical cell type influences the development and progression of breast cancer, with implications for future prevention and treatment strategies targeting macrophages. In this study we evaluated the role of the critical find-me signal receptor P2Y2 on mammary tumorigenesis in a mouse model of breast cancer. The findings from this study are inconclusive regarding the importance of P2Y2 in tumor formation, owing chiefly to the limited number of animals analyzed to date. Future studies will be needed with more robust sample sizes in order to determine significance of this receptor in mammary tumorigenesis.					
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Introduction

The phagocytic clearance of apoptotic cells is a highly efficient process that ensures the orderly removal of potentially inflammatory and immunogenic contents of dead cells from the surrounding tissue. The first step in the phagocytic removal of apoptotic cells is the recruitment of competent, motile phagocytes such as macrophages. Cells undergoing apoptosis release so-called “find-me” signals that mediate the recruitment of phagocytes prior to engulfment. It has recently been shown that nucleotides released by dying cells act as a key find-me signal to promote phagocytic clearance of apoptotic cells in vivo. Mice deficient in the ATP/UTP receptor P2Y₂ display impaired recruitment of monocytes and macrophages to apoptotic cells and delayed corpse clearance. Although apoptosis is an ongoing process in tumors, it is not known how nucleotides released by such apoptotic cells control macrophage recruitment or apoptotic cell clearance during tumorigenesis. The aims of this study are designed to directly test the role of nucleotides as apoptotic cell find-me signals in the recruitment of macrophages to developing mammary tumors. The primary hypothesis is that the efficient recruitment and clearance of apoptotic cells by macrophages reduces inflammation caused by potentially necrotic cells that can spur tumor growth.

Body

Task 1: Generation of mice for studies

Goals:

The goal of Task 1 was to generate mice on the correct genetic background and in sufficient numbers to carry out analysis of the role of find-me signals in tumorigenesis described in Task 2. Specifically, we aimed to generate two different genetic “lines” of mice as outlined below:

“Line 1” – MMTV-PYMT+ males crossed to wild-type females to obtain sufficient numbers of newborn MMTV-PyMT mice with which to track and analyze mammary tumors over time.

“Line 2” – The objective for this line is to obtain mice bearing the MMTV-PYMT transgene on a P2Y₂ heterozygous background (P2Y₂^{+/-}) or a P2Y₂ null background (P2Y₂^{-/-}).

Methods:

Mouse strains. Mice expressing the polyoma middle T antigen under control of the mouse mammary tumor virus (MMTV-PYMT) were previously described¹ and were purchased from Jackson Labs (Bar Harbor, ME). Mice deficient in the P2Y₂ nucleotide receptor (*P2ry2*) were provided by Dr. Beverly Koller at the University of North Carolina². Mice were bred according to University of Rochester Animal Care and Use Committee guidelines in specific pathogen free room.

Breeding strategy. The breeding strategy for generating Line 1 mice was fairly straightforward and produced mice with the MMTV-PYMT transgene. These mice were subsequently analyzed for breast tumors as described below. The breeding strategy for Line 2 mice (P2Y2/MMTV-PYMT) required two crosses. First, P2Y2^{+/+}, MMTV-PYMT⁺ males were crossed with P2Y2^{-/-} females to obtain offspring with the desired genotype (P2Y2^{+/-}, MMTV-PYMT⁺). These pups were then used as breeders in the second cross in order to produce P2Y2^{+/-}, MMTV-PYMT⁺ (as controls) and P2Y2^{-/-}, MMTV-PYMT⁺ as the experimental group. For all mice, at approximately 21 days postnatal, offspring were tagged and genotyped by PCR using genomic DNA from tail biopsies.

Genotyping. Offspring were screened for presence of the PYMT transgene using the PCR-based screening strategy recommended by Jackson Labs. The P2Y2 locus was screened using the previously reported PCR-based strategy², where bands of 414 and 598 base pairs corresponding to wild-type and knockout alleles, respectively, were resolved by agarose gel electrophoresis (Figure 1).

Outcomes:

We successfully generated both of the lines of mice described above. Representative results from PCR genotype analysis in Figure 1 shows that we were able to obtain mice for Line 1 and for Line 2. Mice with the desired genotype were subsequently entered into the study as described in Task 2.

Difficulties:

There were no significant difficulties in carrying out the breeding or obtaining the necessary genotypes for these studies. However, our timing for this breeding was delayed by approximately 6 weeks while waiting for the necessary animal protocol approval to initiate these studies.

Task 2: Quantification of mammary tumors

Goals:

The goal of Task 2 was to use the mice generated in Task 1 to determine the effect of disrupting find-me signal pathways on breast tumors over time using several metrics,

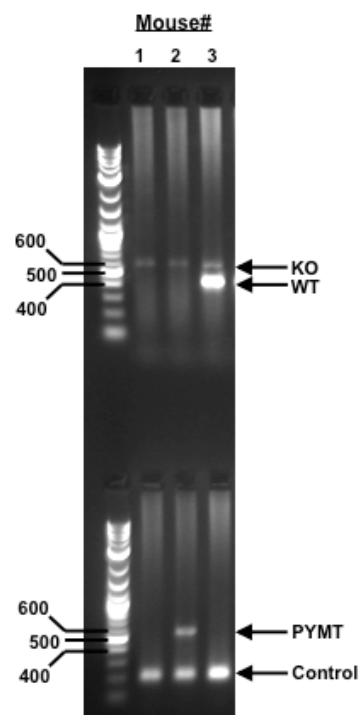


Figure 1. Genotyping results. A representative agarose gel showing PCR analysis of genomic DNA from three Line 2 mice. *Top*, screening for P2Y2 alleles (KO=598bp, WT=414bp). *Bottom*, screening for MMTV-PYMT transgene and an internal positive control (PYMT=556bp, Control=200bp). Markers for specific base pair (bp) sizes are shown to *left*.

including the time to first palpable tumor and total tumor burden.

Methods:

Mice with the correct genotype were entered into the study once they were 6-7 weeks of age and subsequently monitored for tumor growth. The 6-7 week age to begin monitoring was chosen based on preliminary studies discussed below. Mice in the study were monitored as follows. First, each mouse in the study was given a unique identifying number by application of a numbered ear tag. Mice in the study were housed with their siblings (from weaning) to minimize disruptions due to stress from changing cages or cage mates. Mice were palpated every 5-7 days in the chest and abdominal regions to detect tumors. Palpable masses were noted for their position on the ventral side. Measurements were taken of each mass using digital calipers once the mass reached at least 1.0mm in one of the three dimensions measured, length, width or height. Mice were sacrificed when they reached maximum allowable tumor burden or when tumors caused mice to display any difficulty in ability to ambulate, groom or reach food and water. These studies were carried out with conceptual and technical support from the Translation Research Core Facility in the Wilmot Cancer Center at the University of Rochester Medical Center.

Outcomes:

Our discussions with the Translation Research Core Facility led us to take a two-part approach to studying the role of find-me signals in breast tumorigenesis. First, it was necessary to establish baseline metrics for tumor formation and growth in the MMTV-PYMT mouse model. The second part of this study was to analyze the role of the critical find-me signal receptor, P2Y2, in breast tumor formation in this model system.

Tumor formation in MMTV-PYMT mice. The mice used for this portion of the study were from Line 1, and thus their only specific genetic alteration was the presence of the MMTV-PYMT transgene. We began by analyzing mammary tumor growth in females and males in order to compare our colony with previously published reports¹. As shown in Figure 2, we found that the average postnatal age at the time of palpable tumor was 50 days (± 6 days) for females, and 119 days (± 19 days) for males. Thus while males did develop tumors, they required on average 2.38-fold longer to do so. These findings are very similar to those of Guy *et al*, who reported a 2.44-fold longer time to tumor onset in males compared to females for the same transgenic strain¹. However, the average of 50 days to palpable tumor for our mice was 16 days longer than Guy *et al*. Next we focused on the kinetics of

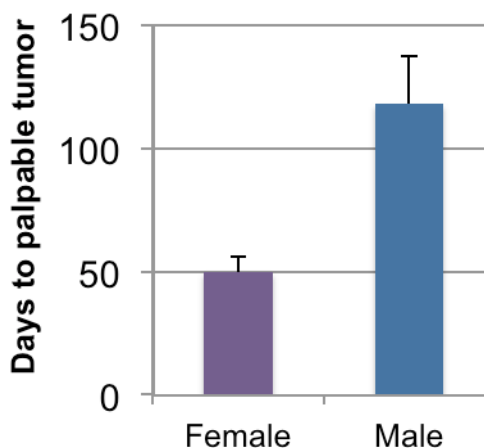


Figure 2. Time to tumor onset in MMTV-PYMT mice. Average days to first palpable mammary tumor in female (n=3) and male (n=9) mice.

tumor formation in female MMTV-PYMT+ mice. While most mice showed a dominant (or primary) tumor, all mice examined showed multiple distinguishable foci of tumor growth (up to 14 in total) in the ventral area over the course of the study. For this reason, we chose to measure total tumor burden by summing the total volume of all measurable tumors on each mouse. When we examined tumor grown in female MMTV-PYMT+ mice up to 100 days postnatal, we found that the kinetics of total tumor burden were somewhat variable as shown in Figure 3. Based on these results, we chose to focus on the onset to first palpable and measurable tumor for our future studies of tumorigenesis in this model.

Tumor formation in P2Y2-deficient mice. Our next objective was to determine the importance of the purinergic find-me signal receptor P2Y2 in the onset of tumor formation in these mice. For this, we entered two groups of MMTV-PYMT+ mice into the study: Control (P2Y2+/-) and Experimental (P2Y2-/-). Our rationale for choosing P2Y2+/- (instead of P2Y2+/+) as controls to compare to P2Y2-/- was that, 1) we have found indistinguishable functional outcomes in macrophages from wild-type and heterozygous P2Y2 littermates³, 2) as Line 1 mice (MMTV-PYVT+ mice) are in fact wild-type at the P2Y2 locus, results from these mice can be compared to heterozygous mice from Line 2 studies to reveal potential differences in tumorigenesis in between wild-type and heterozygous mice, 3) in order to obtain P2Y2 wild-type and P2Y2-/- littermates, it would be necessary to breed MMTV-PYMT+ females in order to transmit a null P2Y2 allele to offspring. This approach would be problematic as female mice display difficulties nursing their pups¹ and would need to be sacrificed after one litter due to the rapid onset of mammary tumors, thus requiring many more breeder pairs to achieve the same number of the desired offspring. For these reasons we have established the two groups of mice, with n=6 in control group and n=9 in the experimental (P2Y2-/-) group, and have monitored these mice for the onset of tumor growth. To date, four of the fifteen mice have developed palpable tumors, two from each group, and the results are shown in Figure 4. There is no statistical difference between these groups ($p=0.6$).

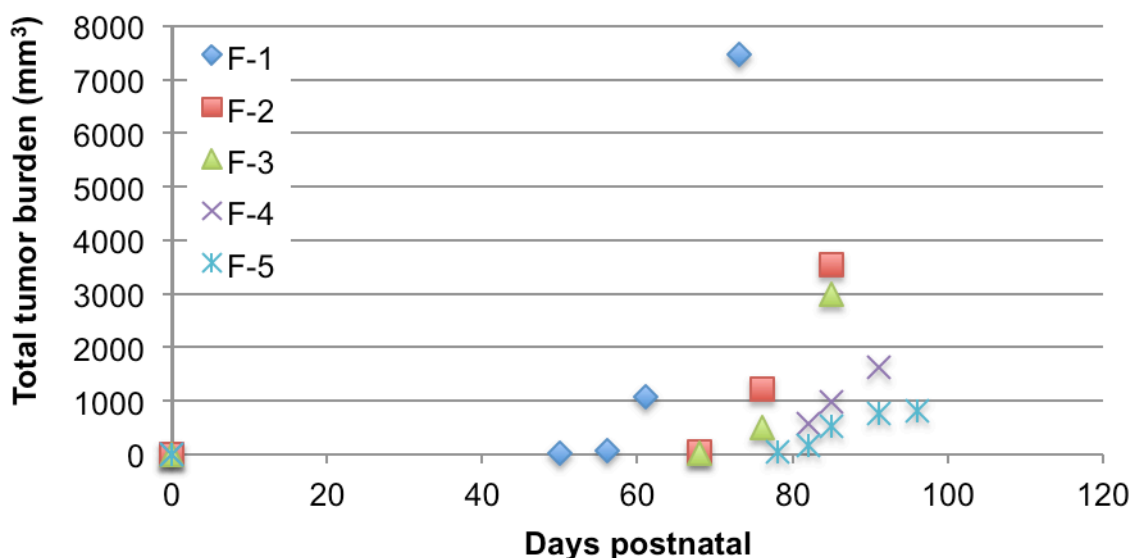


Figure 3. Kinetics of mammary tumor growth. Total tumor burden of female MMTV-PYMT+ mice (n=5) was measured for up to 100 days postnatal.

Difficulties:

Due to the small sample size of mice analyzed thus far, we are unable to make a definitive conclusion regarding the role of the P2Y2 receptor in mammary tumorigenesis. There are both biological and practical reasons for this outcome. The first is that, as shown in Figure 4, the time to onset of palpable tumor in the Line 2 group is thus far nearly double the time we and others^{1,4} have observed in the stock strain of MMTV-PYMT+ mice. We anticipated this analysis to be completed by the end of June 2012, but the additional approximately 7 weeks to onset has prevented this. The second reason, a practical one, is that due to a ~6 week delay in animal protocol approval, the breeding was delayed.

Task 3: Analysis of cell populations in solid tumors

Goals:

The goal of Task 3 was to obtain tumor samples from MMTV-PYMT+ mice in order to assess phagocyte populations at the single cell and tissue level.

Methods:

Tumor bearing MMTV-PYMT+ mice were sacrificed and primary tumor and lung tissues harvested. A portion of the tumors were fixed in formalin for histochemical analysis, while the remaining tissues were dissociated over a mesh filter and trypsinized to obtain a single cell suspension. Cells were then cultured in DMEM 10% heat-inactivated FBS at 37°C, 5% CO₂ in order to enrich for adherent phagocyte and epithelial populations. Fixed tissues were paraffin embedded and mounted on slides for analysis.

Outcomes:

In order to analyze tissues from mammary tumors and metastatic lung tumors, we harvested tissues from mice that had a sufficient mammary tumor burden to correlate with metastasis. Previous reports of the MMTV-PYMT strain have shown that lung metastasis in these mice occurs relatively late in the mammary tumorigenesis process¹, and is associated with high mammary tumor burden. However, we did not observe tumors in the lungs of MMTV-PYMT+ mice. We were able to obtain single cell suspensions of the primary tumors from these mice, and attempted to generate primary tumor cell cultures for analysis, but were unable to due to excessive levels of necrosis in the late stage primary tumors. Similarly difficulties with necrotic tissues were encountered with attempts at whole tissues analysis, although we were able to generate

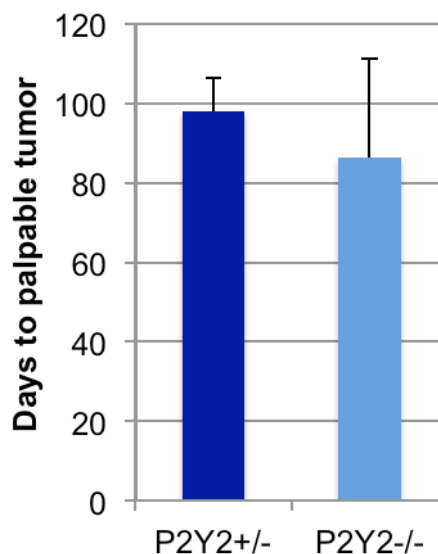


Figure 4. Mammary tumor onset in P2Y2-deficient mice. MMTV-PYMT+ on either P2Y2 heterozygous (n=2) or knockout (n=2) backgrounds were analyzed for onset of mammary tumors.

slide-mounted fixed tumor tissues for portions of primary tumors as well as secondary tumors that were less advanced. Our analysis of these tissues is ongoing.

Difficulties:

As mentioned above, our strategy for this analysis was to examine mammary and lung tumor tissues from the same mice. However, in order to do so, we judged that it would be necessary to allow the primary tumor to grow sufficiently to increase the likelihood of lung metastasis from the primary tumor site. However, this strategy did not yield the desired results for either tissue, as we were unable to detect lung tumors in these mice and the primary tumor had become quite unhealthy and was difficult to process for single cell analysis or to maintain in a healthy culture for any length of time. Based on this, it seems that uncoupling the lung and mammary analysis (that is obtaining different tissues from different mice) would be a strategy more likely to succeed in the future.

Key Research Accomplishments

- We have now established approaches for monitoring and measuring spontaneous mammary tumor growth. Use of this and other spontaneous tumor models are quite uncommon at our institution, thus we have been able to work with the core facility to develop this model.
- We initiated collection of a dataset for MMTV-PYMT mice tumor onset and growth, including palpation, localization, caliper measurement.
- We initiated the acquisition of a data set for onset of tumor formation in mice deficient find-me signaling.

Reportable Outcomes

- Generation of an MMTV-PYMT transformed cell line generated from secondary mammary tumor.
- Employment opportunity: this award occurred prior to accepting a faculty position at the University of Rochester.
- I have since obtained extramural funding from the Ellison Medical Foundation (\$400,000 over 4 years), and the NIH-funded Center for AIDS Research/Creative and Novel Ideas in HIV Research (\$453,000 over 2 years).

Conclusion

Macrophages play a prominent role in tumorigenesis and metastasis, and thus it is critical that we unravel their functional properties that contribute to this. This project was aimed at examining one of the fundamental properties of macrophages- their ability to detect and clear dying cells. Based on previous work, we have sufficient evidence to pursue a specific signaling pathway shown to be important for these functions- the purinergic / P2Y2 signaling pathway. The outcome of our efforts to date have yet to provide the core answers we seek, but the work completed to date has established for the first time (to our knowledge) an effort to understand this aspect of macrophage function in the context of this important and all too common human disease.

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